Ph.D Synopsis

Optimized production and characterization of *in vitro* shoots of *Withania somnifera* as alternate to field grown tissues

Submitted
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Introduction

Medicinal plants play an important role in maintaining good health in the developing countries. Most of the advanced medications available today are derived from the traditional medicinal herbs which satisfy the primary health care needs of nearly three quarters of the world population. As per the report of Gragg *et al*, nearly 30% of the drugs contained the natural products and their derivatives. These herbal remedies and their phytochemical constituents are being studied extensively for the development of nutraceutical compounds against various diseases (Martins and Brijesh, 2018).

Withania somnifera, commonly called as Indian ginseng or ashwagandha is one among the many medicinally important plants in the traditional medicinal system of India. Besides an array of therapeutic properties, ashwagandha is referred to as an adaptogenic herb known to enhance the health and well being mainly by reducing the complications of stress (Lopresti *et al*., 2019). The therapeutic potential of this herb is attributed to a group of 28 carbon containing steroidal lactone compounds referred to as withanolides. The multi-therapeutic potential of this plant as well as the individual withanolides has resulted in a significant demand for its raw materials in the pharmaceutical industry. Furthermore, due to the low viability and germination potential of the seeds, traditional propagation of this plant is quite difficult. It should also be mentioned that withanolide accumulation in various parts of the plant is extremely low, ranging from 0.001 to 0.5 percent. Thus for the commercial production of withanolides, plant tissue culture is strongly suggested for multiplication of this plant, especially because the metabolite of interest is tissue specific. Withania somnifera, being a plant with high metabolic content can be considered as a recalcitrant species with a high capacity for *in vitro* proliferation (Yildiz, 2012). Therefore, a variety of strategies including elicitation are being critically examined for mass production and increased metabolite accumulation in *in vitro* cultures (Sabir *et al*., 2013). Elicitors being factors or compounds that trigger the defence response in plants leading to increased secondary metabolite production also increases the biological activity of the plants (Jonathan *et al*., 2015). Seaweeds with reported applications in food and cosmetic industry is also said to pocess biostimulation effect on plants thus serving as a good soil conditioner and biofertilizer. Various bioactive components in the seaweeds like ulvans, carrageenans, alginates and laminarin have also been reported to enhance the disease resistance in plants by triggering the defence response (Pettongkhao *et al*., 2019). The seaweed strain *Kappaphycus alverizii* as reported by Mani *et al*

(2021), upregulates the disease responsive genes besides increasing the plant biomass and reduces the use of chemical fertilizer by 50% . Fungal mycelia extracts as well as culture filterates have also shown reportedly shown to increase biomass and withanolides content in *Withania somnifera* cultures (Ahlawat *et al*., 2017).

Withanolides being the legendery bioactive components are distributed in a tissue specific manner, thus the in vitro culture of identified tissue is an alternate choice to augument the withanolide production. Among nearly 4000 withanolides studied so far, withanolide A and withaferin A are considered as dominant over the others. Earlier studies have also reported the leaf tissues to accumulate withaferin A in higher proportions and the root tissues to be a repository of withanolide A (Thirugnanasambantham *et al*., 2014). Further the activity assessment of the bioactive withanolides withanolide A, withaferin A and withanone in suppressing the cytokine production in LPS induced inflammatory macrophage cell line showed withaferin A to be more potent at very low concentrations in suppressing cytokine production than the other withanolides. Further the same study involving the *in vitro* and field grown extracts of *Withania somnifera* also reported *in vitro* shoots of *Withania somnifera* to be equally potent as that of the field grown roots in suppressing cytokine production (Purushotham *et al.*, 2017). Whereas, the *in vitro* roots showed no such activity which can be related to the trace accumulation of withaferin A in these tissues (Senthil *et al*., 2015). These studies have led to the hypothesis that withaferin A is the potent bioactive compound that acts synergistically with other withanolides. Based on the above reported information the present study is designed:

1. To improve the yield and validate the potential of *in vitro* shoot cultures as an alternate to traditionally used field grown roots.

The strategies followed to meet the objectives are as follows:

1. Standardisation of the best elicitation condition for maximum biomass yield and withaferin A content in *in vitro* shoots of *Withania somnifera*.
2. To study the effect of elicitors on biomass yield and metabolite content under different growth conditions
3. To study the metabolic profile of the *in vitro* shoots grown under standardized conditions in comparison with field grown tissues
4. To screen for the oxidative stress targets of withanolides using *in silico* tools and it's *in vitro* validation.

Materials and methods:

Collection of ligands and ADME profiling:

The 3D structures and canonical SMILES of the ligands withanolide A, withanolide B, withanolide D, withanolide E, withanolide F, withaferin A and withanone were obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in their SDF formats. The SDF files were then converted to PDB files using Open Babel GUI. The Adsorption, Distribution, Metabolism, Excretion and Toxicity profiling of the metabolites were done using the freely accessible web tools Swiss ADME (http://www.swissadme.ch/) and pkCSM- pharmacokinetics (http://structure.bioc.cam.ac.uk/pkcsm). Both the tools makes use of the canonical SMILES as the input file for the prediction of physicochemical and ADMET properties.

Prediction of biological activities:

The SMILES file was used as an input to predict the spectrum of biological activities of each compound using PASS online version (http://way2drug.com/PassOnline/.php). This program anticipates the probability of biological activities of compounds with 90% accuracy. For any compound an activity is considered possible only when the Pa> Pi, where Pa is the probability of active compound and Pi is the probability of inactive compound. Only activities with Pa> 0.7(high reproducibility experimentally) were filtered.

Prediction of targets of withanolides:

The retrieved SMILES file of each withanolide served as input file for the free online databases "Swiss target" (http://www.swisstargetprediction.ch/) and "DIGEP-pred" (http://www.way2drug.com/ge/) for the identification of potentially active targets whose biological activities were found to have Pa value greater than 0.5. The target genes retrieved from both these databases were then pooled together to obtain the final targets of each of the withanolide.

Prediction of oxidative stress targets of withanolides:

The oxidative stress related target genes were extracted from AmiGO2 database version 2.1.15 (http://amigo.geneontology.org/) (Carbon et al., 2009). By using 'oxidative stress' as the key word, nine gene ontology (GO) terms related to oxidative stress were obtained. The gene targets retrieved from these nine target GO data sets were pooled and all duplicates removed to

obtain the final list of oxidative stress related target proteins. Following this the oxidative stress targets of each of the withanolide were obtained by overlapping the oxidative stress targets with that of the withanolide targets by constructing venn diagrams using FunRich software (Fonseka et al., 2021). The gene set obtained as anti-stress targets of each particular withanolide were then pooled to obtain the final oxidative stress targets of the withanolides. The GO enrichment analysis of the obtained final target hits were carried out using Funrich software based on the go terms: cellular component, molecular function and biological process. Each of the enriched GO terms was plotted as graphs of the top 10 enriched terms.

Protein protein interaction (ppi) network construction:

The finally obtained target set of oxidative stress targets of the withanolides were investigated for its protein –protein interaction (ppi) using STRING (https://string-db.org/) for Homo sapiens (Szklarczyk et al., 2019). The ppi network was further subjected to pathway enrichment based on the confidence score. The pathway enrichment was done in relation to the KEGG pathway database. Among all the enriched pathways, the highly enriched pathways related to oxidative stress as obtained from previous literature were chosen for further analysis. Among them the hub genes with maximum interaction with other gene/ proteins were screened.

Molecular docking:

The finally screened targets from the network analysis were then evaluated by molecular docking. The 3D structures of the hub protein targets obtained were retrieved from protein data bank (PDB) (https://www.rcsb.org/). The water molecules and ligands associated with it were removed using Pymol 2. Further the protein preparations were done by addition of charges and polar hydrogens using Autodock tools followed by a rigid docking against all the six withanolides individually using Autodock 1.5.6. Among the ten binding poses obtained, the one with the least binding energy was considered as the best docking confirmation. The binding pockets and the interacting residues were visualized using Discovery studio Biovia 2020.

Plant material:

The seeds of *Withania somnifera* Jawahar -20 were obtained from Central Institute of Medicinal and Aromatic Plants (Lucknow). The overnight soaked seeds were germinated in half strength MS (Murashige-Skoog) media amended with 2% sucrose and maintained under complete darkness. The germinated seedlings were then transferred to full strength MS basal medium and maintained on 16/8 hours photoperiod at 22 ± 2^0C with regular sub culture. The healthy fresh nodes

from two month old MS grown *in vitro* plants were inoculated in MS solid media containing 4.44µM Benzylaminopurine (BAP) for shoot multiplication as reported in our previous study (Parameswari et al., 2017). One month old shoots from MS+BAP medium were used for the elicitation study.

Standardisation of seaweed and fungal elicitation in *in vitro* cultures of *Withania somnifera*:

For elicitation of biomass in *in vitro* cultures of *Withania somnifera* two biotic elicitors: a seaweed extract and a fungal mycelia extract were chosen. A commercially available seaweed extract powder containing *Kappaphycus alverzii* species were obtained from HVG Industries, Chennai. The fungal mycelia mat obtained from *Aspergillus niger* cultures was extracted in water, sterilized and stored. Both the seaweed extract (%w/v) and mycelia extract (% v/v) at different concentrations (0.15%, 0.25%. 0.50 %, 1% and 2%) were added to the MS media and pH adjusted to 5.6-5.8 prior to mixing with agar. The one month old proliferated shoot cultures of *Withania somnifera* from MS-BAP media weighing approximately 2-3g with 1-2 nodes were subjected to elicitation studies. The elicitation was carried out for different durations like 6hrs, 12hrs, 24hrs, 48 hrs, 7 days and 14 days for standardisation of duration of exposure. Based on the preliminary standardisation studies, the dual elicitation effect of the combination of these two elicitors were carried out using lower concentrations of both the elicitors for the appropriate exposure time. Each elicitation were done in triplicates and maintained at 22 ± 2^0C under 16/8 hr photoperiod. The elicited shoots after the respective exposure times were again maintained in MS+ BAP media until harvest after 14 days. The best among the elicitation conditions were chosen based on the maximum biomass and withaferin A accumulation compared to the control.

Influence of growth conditions on the biomass and withaferin A content in *in vitro* shoots of *Withania somnifera*:

The control along with the three best elicitation conditions obtained using seaweed extract, mycelial extract and dual elicitation were chosen for the study of influence of growth conditions. The same elicitations were performed in semi- solid, suspension and bioreactor cultures. For both suspension and bioreactor cultures, the elicitation were carried out in liquid MS media along with a control. For suspension cultures, the culture vessels were kept in an orbital shaker under continuous agitation at 80rpm. Whereas for bioreactor cultures a bubble column bioreactor (Biopia, Republic of Korea) fitted with a 0.02µm hydrophobic membrane filter (Sartorius stedim) was used under continuous aeration of 0.1vvm using an mini aerator.

Determination of growth index:

The biomass of the harvested shoots after each treatment was calculated as the difference in weight between the initial and final weights. From this the growth index (GI) was also calculated using the formula:

GI = ((Harvested fresh weight- Inoculated fresh weight) / Inoculated fresh weight) * 100

Preparation of extracts:

The control and elicitor treated shoots after harvest were rinsed and blotted to remove extra water, air dried and powdered in a mortar and pestle. One gram of the shoot powder was subjected to methanolic extraction. Each sample was extracted sequentially four times at a sample to solvent ratio of 1:50 throughout the extraction. The sample solvent mixture was sonicated for 20mins before each extraction and placed in a shaker for 2hrs at 100rpm. At the end of each extraction, the extract was filtered using whatmann No. 1 filter paper, pooled and concentrated on a rotary evaporator at 125rpm in a water bath at 40°C. The final residue was finally dissolved in 10ml of HPLC methanol and stored at -20°C for further use.

Preparation of standards

Standard Withanolide A and Withanone were obtained from chromodex (USA). Withaferin A was obtained from natural remedies (Bangalore). Stock solutions of the standards were prepared in concentration of 1mg/ml using HPLC methanol and stored at 4°C. Before use, the working standards were prepared by dilution of the stock to a final concentration of 0.5mg/ml.

HPTLC Quantification of Withanolides

High Performance Thin layer chromatography (HPTLC) quantifications were carried out in a solvent system containing toluene, ethyl acetate and formic acid in 5:5:1 (v/v) ratio respectively. The procedure was carried out on silica precoated aluminium plates of 0.2mm thickness. The standard withanolide-A, withanone and withaferin-A were applied in the concentration ranging from 0.5-2.0 µg per band for quantification. The samples prepared in HPLC methanol were spotted on the plates along with the standards as 6 mm bands marked 8mm from the bottom. The samples were applied by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator employed with a 100µl Hamilton syringe working under a stream of nitrogen (spraying rate: 150nLs^{-1}). The loaded plates were developed upto a distance of

80mm in a presaturated twin trough chamber, air dried and visualized at 254 and 366nm. Densitometric scanning was carried out using Camag TLC scanner III controlled by CAMAG CATS 4 integration software operating with Savitsky-Golay7 filter in the reflectance –absorbance mode at 223nm for withaferin A, 234nm for withanolide A and 231nm for withanone respectively at a scanning speed of 20 mm s^{-1}. The resolved spots in the samples were compared with the Rf values and peak areas of the standards. The plates were then derivatized using Anisaldehyde sulphuric acid reagent and kept in hot air oven at 110°C for about 10min for detection of spots (Preethi *et al*., 2014).

GC-MS Analysis:

100 µl of the methanolic extracts were vacuum evaporated and the dried residues ware incubated at 37^0C with 50 µl of methoxylamine hydrochloride (20mg/ml pyridine) for 2 hrs. The acidic protons formed were derivatized at 37^0C for 30 min using 50 µl of MSTFA (N-methyl N-trimethyl silyl trifluoroacetamide) and used for metabolite profiling. The gas chromatography was performed on a 0.25µm thick Rxi-5 silicon MS column 30m long and 0.25mm in diameter. The sample injection was carried out with an ion source at a temperature set to 250^0C with helium flow adjusted to 1ml min^{-1}. After an initial solvent delay time (8 min at 50^0C), the column temperature was programmed to increase at the rate of 6.5^0C min^{-1} upto 300^0C with 5 min hold time. Each 1 µl aliquot of the derivatized extracts were injected onto the GC/MS system (shimadzu 2010 plus) with a split ratio of 1:10 after tuning of the system as per the manual with helium as a reference gas. The high mass resolution was improved manually to reach maximum resolution of R_{fwhm}=2800 at 614 m/z. The mass spectra were observed between 45m/z and 650 m/z with scanning speed of 0.5s $scan^{-1}$. The interpretation of the GCMS data obtained was done using AMDIS software (version 2.62). The peaks obtained were confirmed against the two spectra libraries NIST 05 (https//:chemdata.nist.gov) and GOLM metabolome database (gmd.mpimp-golm.mpg.de). The relative amount percentage of the metabolites were extracted and subjected to Principal component analysis (PCA).

Maintenance of cell lines:

The raw 264.7 cell lines (murine macrophage) were obtained from NCCS, Pune and maintained in DMEM (Dulbecco's Modified Eagle's Medium) containing high glucose and supplemented with 10% FBS and 1% antimycotic solution. Further the culture conditions were set to 5% CO_2, 18-20 % O_2 at 37^0C in a CO2 incubator with regular sub-culturing every two days.

MTT assay:

200µl of the cell suspension containing viable cells at a density of $2X10^4$ were seeded onto 96 well plates and incubated for 24 hrs for the formation of monolayer. Appropriate concentrations of the standard and plant extracts were added and incubated at 37°C in a 5% CO_2 atmosphere. After 24 hrs, the extra media was removed and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was added to a final concentration of 0.5mg/ml. The MTT treated plates were wrapped with aluminium foil and incubated for further three hours. The supernatant was removed and 100 µl of DMSO (solubilisation solution) was added and shaken to solubilize the formazan crystals. The plates were then read at 570nm on an ELISA reader. The cell viability was then calculated using the below formula:

% cell viabilty = (Absorption of treated cells/absorption of untreated cells) X 100

The IC_{50} values were determined by using linear regression equation i.e. Y =Mx+C.

where, Y = 50, M and C values were derived from the viability graph.

The cultured macrophage cell lines were treated with LPS at a concentration of 10µg/ml for 3hrs to induce toxicity and then exposed to desired concentrations of the plant extracts and incubated for further 24 hrs. An untreated well served as a control and subjected to analysis by flow cytometry.

Apoptosis assay:

Toxicity was induced in the macrophage cell lines (0.5×10^6 cells/2 ml) cultured overnight in a 96 well plate by exposing to LPS (10ug/ml) for 3hrs. The induced cells were then treated with 100 µl of FR, BS and 10 µl of standard withaferin A individually in each wells and incubated for 24 hours. The anti-apoptotic activity of the extracts was determined by Annexin V FITC/ Propidium iodide binding assay. In brief, the treated and untreated cells were washed with PBS and harvested. The harvested cells were then incubated at 370C for 3-4 mins with 200ul of trypsin-EDTA solution. The cells were then subsequently treated with 5ul of FITC, 5ul of PI and 400 ul of 1X binding buffer, vortexed and then analysed by flow cytometry.

ROS assay:

The differential ROS scavenging capability of the *in vitro* shoot and field root extracts were studied using the fluorescent dye H2DCFDA (2'-7'- dichloro dihydrofluorescein diacetate) on LPS induced macrophage raw 264.7 cell lines. The cells were cultured in a 6 well plate at a density of 0.5 X 10^6 cells /2ml. The cells were then stimulated with LPS at a concentration of 10µg/ml for 3 hrs and incubated overnight after treating with the 100 µl of the plant extracts. At the end of incubation, the cells were suspended in H2DCFDA solution and incubated for further 30 min at 37^0C away from light. The treated cells were then washed in PBS and the fluorescence of the cells was measured at an excitation and emission wavelength of 488nm and 535 nm respectively in a flow cytometer. The BD FACS Calibur employed with Cell Quest software (version 6.0) was used to refine the M1 and M2 gating phases.

DNA damage:

The amount of DNA damage on apoptosis was detected by the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) assay. The LPS and extract treated cells after overnight incubation were suspended at a concentration of 1X10^6 cells/ml in 1%(w/v) paraformaldehyde in PBS (pH 7.4) and cooled over ice for 30-60 mins. The cells after further buffer wash were resuspended in 50ul of DNA labelling solution containing TdT enzyme and FITC dUTP in the reaction buffer and incubated at 37^0C for 60 mins.at the end of incubation, the cells rinsed twice with the buffer and resuspensded in PI/RNase staining buffer and incubated at room temperature in dark for 30 mins and analysed in a flow cytometer (BD FACSCalibur, BD Biosciences, CA, USA) within 3hrs.

Statistical analysis:

All the experiments were performed in triplicates. Data were statistically analyzed using ANOVA and DMRT multivariate test in SPSS. The XL STAT plug-in (Addinsoft, New york, USA) in MS excel was used in the principal component analysis of the data.

Results

1. The comparative ADMET profiling of withaferin A with the other withanolides of importance namely withanolide A, withanolide B, withanolide D, withanolide E and withanone showed all the six withanolides to pocess good drug like properties showing no violations against the lipinski's rule of five. Though all the withanolides studied were slightly toxic and tolerable at very low doses, they showed no skin sensitisation, hepatotoxicity or mutagenecity thus revealing their drug likeliness.
2. The mining of target genes of each of the withanolides using Swiss target and Digeppred target database yielded a total of 317, 313, 336, 228, 273 and 289 gene targets for withanolide A, withanolide B, withanolide D, withanolide E, withaferin A and withanone respectively. Further the screening of gene targets related to oxidative stress from the AMIGO2 database yielded nine gene ontologies related to oxidative stress contributing to a total of 572 target genes.
3. The common genes among the oxidative stress target dataset with each of the withanolide target datasets was retrieved by construction of venn diagrams using funrich software which were considered as the oxidative stress targets of the particular withanolide. Thus a total of 30, 30, 34, 24, 27 and 31 genes were obtained as oxidative stress targets of withanolide A, withanolide B, withanolide D, withanolide E, withaferin A and withanone respectively. All these target proteins were pooled and duplicates removed to yield a total of 53 oxidative stress targets of the withanolides.
4. The protein -protein interaction among the 53 targets (nodes) showed 242 connections (edges) with ppi enrichment value of $<1.0\ e^{-16}$. The pathway enrichment analysis of these 53 targets on the basis of KEGG pathway database showed the enrichment of the 53 target genes in 112 KEGG pathways among which AGE RAGE signalling pathway was highly enriched with false discovery rate of $6.81\ e^{-09}$. Thus the AGE RAGE signalling pathway and its underlying pathways namely MAPK signalling pathway, FOXO signalling pathway and PI3-AKT signalling pathway which are all involved in oxidative stress response were selected. Thus the

dataset of 53 oxidative stress targets were screened out to 16 protein targets involved in the above selected four pathways.

5. Further the screening of the hub genes among these 16 targets showed BCl2 and MAPK8 to be the hub genes that interacted well with the other targets and frequently occurred among the four pathways. Withaferin A showed the best docking score amongst all the withanolides against MAPK8 (binding energy: -10.15). Whereas, docking with BCl2 revealed other withanolides to be more potent with least binding energy revealing the synergistic effect of the withanolides towards BCL2.
6. The initial standardisation of elicitation conditions using *Aspergillus niger* mycelial extract at different exposure times involving different concentrations of the elicitor showed an linear increase in the growth index and withaferin A content along the increase in duration of exposure upto seven days. An increase in exposure time above seven days showed a downfall in both the growth index and withaferin A content. Among the different concentrations of the elicitors used, shoots treated with 0.25% of the elicitor exhibited maximum growth index and withaferin A content. Thus 0.25% of the elicitor at 7 days exposure time yielded maximum growth index (2.38 ± 0.3) and withaferin A content (1.28 ± 0.1 mg/g DW) which is 1.27 and 1.52 fold greater than the control.
7. The elicitation of the shoots with seaweed extracts also showed similar increase in growth index and withaferin A content upto seven days of exposure with maximum yield in 0.25% elicitor treated shoots. Thus 0.25% of seaweed elicited shoots with 7 days exposure time showed maximum growth index (3.67 ± 0.7) and withaferin A content (2.52 ± 0.1mg/g DW) which is 1.91 and 3.1 fold greater than the control.
8. Since both the elicitors showed good yields on 7 days exposure at lower concentrations only lower concentrations of the elicitors for seven days exposure time were used to study the combined effect of these elicitors. Among the dual elicited groups, the combination of 0.25% of seaweed extract with 0.25% of the fungal mycelial extract produced maximum growth index (3.04 ± 0.2) and withaferin A (2.01 ± 0.2) content which was 1.65 and 2.30 fold greater than the control.
9. All the above three best elicitation conditions (ie: 0.25% seaweed extract, 0.25% mycelia extract and its combination) were used along with a control to study the effect of growth conditions (solid, suspension and bioreactor) on growth index and withaferin A content. The advancement in culture techniques from solid agar to liquid suspension cultures to large scale

bioreactor cultures showed a linear increase in the growth index irrespective of the elicitor used.

10. Also among different elicitation conditions, 0.25% seaweed treated shoots showed maximum biomass accumulation in all the three growth conditions. Thus, the seaweed treated shoots in bioreactor exhibited maximum growth index of 7.32 ± 0.2 which was 2.59 fold higher than the bioreactor control and 3.70 fold greater than the Solid control shoots.
11. The withanolides content in the elicited and control shoots in each growth phase as well as field grown root and shoot tissues were quantified by High performance thin layer chromatography (HPTLC) against standard withaferin A, withanolide A and withanone. It was observed that on contrary to growth index, a decrease in withanolides content was observed with advancement in culture techniques. Thus the bioreactor cultures with maximum growth index exhibited minimal withanolide content in terms of mg withanolides per gram dry weight. But taking into consideration the increased growth index the final yields of withanolides were found to be higher in bioreactor cultures compared to solid and suspension cultures. Thus the seaweed treated shoots in bioreactors exhibited the maximum growth index and withanolides content in terms of mg withanolides/ harvest.
12. Thus the seaweed treated shoots in bioreactors exhibited 18.56 fold increase in withaferin A (6.66 ± 0.4), 8.96 fold increase in withanolide A (0.83 ± 0.2) and 7.29 fold increase in withanone (0.74 ± 0.1) content compared to control shoots in solid. Also since a decrease in withanolide content was observed with advancement in culture technique, the seaweed treated shoots in bioreactors expressed lower yield of the withaferin A and withanone content than field grown shoots (6.59 ± 0.2 and 2.04 ± 0.5 respectively). This lower yield in withanolides makes it similar to that of field grown roots which expressed 0.78 ± 0.3 mg/g DW withaferin A and 0.51 ± 0.3 mg/g DW withanone respectively.
13. Further the profiling of bioactive compounds in the methanolic extracts of field shoot, field root and *in vitro* control and seaweed treated shoots in different growth conditions was performed using GCMS. The curation of GCMS data with AMDIS software and retrieval of the peaks with NIST and GOLM library showed the presence of total of 34 metabolites related to withanolide biosynthesis. The metabolites reported include 13 organic acids, 10 amino acids, 3 sugars, 3 alcohols and 4 other compounds.
14. Among the 13 organic acids, the field shoot samples showed high content of propanoic acid, linoleadic acid, oxalic acid, succinic acid, linolenic acid, fumaric acid and valeric acid whereas field roots accumulated high levels of stearic acid and myristic acid. Seaweed

elicitation in *in vitro* shoots showed positive response towards all the organic acid except myristic acid, fumaric acid and levulinic acid.

15. The amino acid proline that accumulates in response to stress was found to be higher in seaweed treated shoots compared to the control. Further, proline was highly accumulated in field shoot compared to field root tissues. Both field shoot and root samples showed higher levels of amino acids asparagine and glutamic acid which were present in very trace quantities in *in vitro* shoot tissues.

16. The sugars glucose and galactose increased on seaweed elicitation and were present in very trace quantities in field tissues. Whereas, mannose levels decreased on seaweed elicitation. The levels of the sugar alcohol, myo-inositol increased on seaweed elicitation as well as with the advancement in culture techniques. Thus seaweed treated bioreactor shoots exhibited higher levels of myo-inositol which were only in trace quantities in field root and shoot tissues.

17. Among the other important compounds, squalene was relatively high in solid seaweed samples. Thus contributing to high withanolide content in seaweed treated shoots in solid culture.

18. The complexity of this GCMS data was further simplified with Principal component analysis (PCA) in which the 34 metabolites studied were plotted against two principal components PC1 and PC2 which contributed to about 85% of the variation in the dataset. The construction of biplot showed a clear distinction between the control and seaweed treated samples along the PC1 which was positively correlated to most of the amino acids and organic acids especially squalene, the precursor of withanolide biosynthesis.

19. Further the distinction amongst the growth conditions from solid to suspension to bioreactor cultures were plotted along PC2 whose loading factors were higher for solid cultures followed by suspension and bioreactor cultures. Thus the clustering of the samples based on the PCA analysis showed the field shoot and root samples to be clustered together and all the *in vitro* shoots to form a separate cluster. Among the *in vitro* shoot samples, the bioreactor grown shoots were clustered closest to the field grown tissues with the solid cultured shoots to be distantly related to the field tissues.

20. The *in vitro* toxicity analysis of the bioreactor control and seaweed treated tissues, field root and shoot tissues along with standard withaferin A showed a significant difference in cell viability in raw 264.7 macrophage cell lines along with increase in concentration of the samples. Withaferin A was found to be toxic at lower concentrations with IC50 value around

84µg. Among the samples tested, field shoot tissues were highly toxic with IC50 value of around 9 mg compared to field root samples with IC50 value around 37 mg. The bioreactor control and seaweed treated shoots showed nearly same IC50 value around 25mg indicating no specific toxicity was produced on addition of seaweed.

21. The intracellular ROS in the cells are capable of oxidising the dye 2'-7'- dichloro dihydrofluorescein diacetate into a fluorescent dichloro dihydrofluorescein which is measured using flow cytometry. Thus the field root samples showed maximum suppression of ROS levels from 83.40 ± 1.9 % in LPS induced group to about 11.92 ± 1.3% followed by standard withaferin A (24.91 ± 2.4%) and seaweed treated samples in bioreactor (38.82 ± 3.1%).
22. Similar suppression of DNA damage was observed in cells treated with standard withaferin A (25µg), field root (5mg) and field shoot (5mg) extracts. A significant difference was observed in the percentage of DNA damage among the sample groups from 68.62 ± 3.5% in LPS induced group to 16.62 ± 3.5%, 14.57 ± 2.2% and 7.64 ± 0.9% in Withaferin A, bioreactor seaweed and field root treated groups.
23. The *in vitro* shoot (bioreactor seaweed treated shoots) extracts showed similar suppression of apoptosis leading to increase in the percentage of live cells similar to that of the field grown root extracts. Thus the 2.56 ± 0.5% live cells in the LPS induced group was significantly increased to 71.81 ± 1.5% in standard withaferin A treated group followed by 80.89 ± 0.5% and 89.72 ± 1.2% in *in vitro* shoot and field root treated groups.
24. Thus this study showed the efficacy of *in vitro* shoots in combating oxidative stress thus preventing DNA damage and apoptosis relative to traditionally used field grown roots. The mechanism of action of the field root and *in vitro* shoots relative to standard withaferin A was studied by the gene expression profiling of the hub genes obtained from in silico analysis (BCl2 and MAPK8). The upregulation of MAPK8 and suppression of BCl2 expression in response to oxidative stress as reflected in the LPS induced group was reversed effectively by the standard withaferin A, field root and *in vitro* shoot samples thus preventing DNA damage and apoptosis.

Conclusion

Thus it can be summarized that withanolide accumulation is significantly influenced by the growth conditions as well as the elicitor used. Seaweeds being organic in nature, the addition of seaweeds in media do not affect the final quality of the product as evident from the toxicity analysis. Further seaweed elicitation has also increased the metabolic content as well as the antioxidant potential of the plant. Thus our results indicate *in vitro* shoot cultures of *W. somnifera* may be used as a valuable alternative raw material in place of traditionally used field grown roots for production of therapeutic formulations. Thus the above study will be useful for the large-scale cultivation of *in vitro* shoots of *Withania somnifera* for the production of withaferin A.

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